

EXHIBIT D

Crystal Structure of the *Aequorea victoria* Green Fluorescent Protein

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The green fluorescent protein (GFP) from the Pacific Northwest jellyfish *Aequorea victoria* has generated intense interest as a marker for gene expression and localization of gene products. The chromophore, resulting from the spontaneous cyclization and oxidation of the sequence -Ser⁶⁵ (or Thr⁶⁵)-Tyr⁶⁶-Gly⁶⁷-, requires the native protein fold for both formation and fluorescence emission. The structure of Thr⁶⁵ GFP has been determined at 1.9 angstrom resolution. The protein fold consists of an 11-stranded β barrel with a coaxial helix, with the chromophore forming from the central helix. Directed mutagenesis of one residue adjacent to the chromophore, Thr²⁰³, to Tyr or His results in significantly red-shifted excitation and emission maxima.

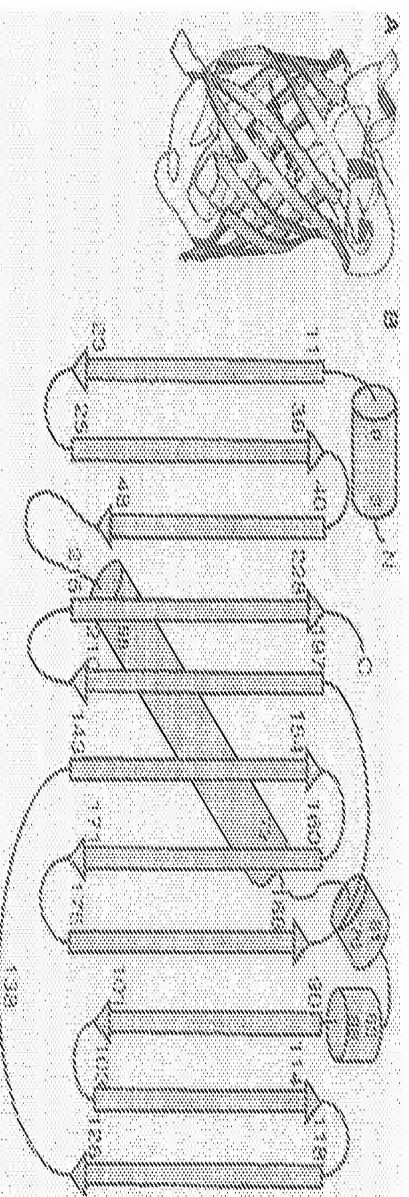
Although the GFP of the Pacific Northwest jellyfish *Aequorea victoria* was discovered some time ago (1), the cloning (2) and heterologous expression (3) of its cDNA were the crucial steps that triggered the widespread and growing use of GFP as a reporter for gene expression and protein localization in a broad variety of organisms (4, 5). Wild-type GFP is a stable, proteolytically-resistant single chain of 238 residues and has two absorption maxima at about 395 and 475 nm. The relative amplitudes of these two peaks are sensitive to environmental factors (6) and illumination history (4), presumably reflecting two or more ground states. Excitation at the primary absorption peak of 395 nm yields an emission maximum at 508 nm with a quantum yield of 0.72 to 0.85 (1, 4–6). The fluorescence results from the autocatalytic cyclization of the polypeptide backbone between residues Ser⁶⁵ and Gly⁶⁷ and oxidation of the α - β bond of Tyr⁶⁶ (4, 7, 8). Mutation of Ser⁶⁵ to Thr (S65T) (9) simplifies the excitation spectrum to a single peak at 488 nm of enhanced amplitude (10), which no longer shows signs of conformational isomers (4). As a step in understanding these properties, and to aid in the tailoring of GFPs with altered characteristics, we have determined the three-dimensional structure at 1.9 Å resolution of the S65T mutant (10) of *A. victoria* GFP (11).

The structure of GFP was determined by

multiple isomorphous replacement and anomalous scattering (11) (Table 1), solvent flattening, phase combination, and crystallographic refinement. The most distinctive feature of the fold of GFP is an 11-stranded β barrel wrapped around a single central helix (Fig. 1, A and B), where each strand consists of approximately 9 to 13 residues. The barrel forms a nearly perfect cylinder 42 Å long and 24 Å in diameter. The NH₂-terminal half of the polypeptide comprises three antiparallel strands, the central helix, and then another three antiparallel strands, the third of which (residues 118 to 123) is parallel to the NH₂-terminal strand (residues 11 to 23). The polypeptide backbone then crosses the "bottom" of the molecule to form the second half of the barrel in a five-strand Greek key motif. The top end of the cylinder is capped by three short, distorted helical segments, and one

short, very distorted helical segment caps the bottom of the cylinder. The main chain hydrogen bonding lining the surface of the cylinder likely accounts for the unusual stability of the protein toward denaturation and proteolysis. There are no large segments of the polypeptide that could be excised while preserving the intactness of the shell around the chromophore. Thus, it would seem difficult to re-engineer GFP to reduce its molecular size (12) by a large percentage.

The *p*-hydroxybenzylideneimidazolidinone chromophore (7) is completely protected from bulk solvent and is centrally located in the molecule. The total and presumably rigid encapsulation is probably responsible for the small Stokes' shift (that is, wavelength difference between excitation and emission maxima), high quantum yield of fluorescence, inability of O₂ to quench the excited state (13), and resistance of the chromophore to titration of the external pH (6). It also allows one to rationalize why fluorophore formation should be a spontaneous intramolecular process (8), because it is difficult to imagine how an enzyme could gain access to the substrate. The plane of the chromophore is roughly perpendicular (60°) to the symmetry axis of the surrounding barrel. One side of the chromophore faces an unexpectedly large cavity that occupies a volume of ~135 Å³ (14). The cavity does not open out to bulk solvent. Four water molecules are located in the cavity, forming a chain of hydrogen bonds linking the buried side chains of Glu²²² and Glu⁶⁹. Unless occupied, such a large cavity would be expected to destabilize the protein by several kilocalories per mole (15). Part of the volume of the cavity might be the consequence of the compaction resulting from cyclization and de-



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Fig. 1. (A) Schematic drawing of the backbone of GFP produced by the program MOLSCRIPT (32). The chromophore is shown as a ball and stick model. **(B)** Schematic drawing of the overall fold of GFP. Approximate residue numbers mark the beginning and ending of the secondary structure elements. N, NH₂-terminus; C, COOH-terminus.

hydration reactions. The cavity might also temporarily accommodate the oxidant, most likely O₂ (4, 8, 16), that dehydrogenates the α-β bond of Tyr⁶⁶. The chromophore, cavity, and side chains that contact the chromophore are shown in Fig. 2A, and a portion of the final electron density map in this vicinity is shown in Fig. 2B. The opposite side of the chromophore is packed against several aromatic and polar side chains. Of particular interest is the intricate network of polar interactions with the chromophore (Fig. 2C). His¹⁴⁸, Thr²⁰³, and Ser²⁰⁵ form hydrogen bonds with the phenolic hydroxyl; Arg⁹⁶ and Gln⁹⁴ interact with the carbonyl of the imidazolidinone ring, and Glu²²² forms a hydrogen bond with the side chain of Thr⁶⁵. Additional polar interactions, such as hydrogen bonds to Arg⁹⁶ from the carbonyl of Thr⁶², and the side-chain carbonyl of Gln¹⁸³, presumably stabilize the buried Arg⁹⁶ in its protonated form. In turn, this buried charge suggests that a partial negative charge resides on the carbonyl oxygen of the imidazolidinone ring of the deprotonated fluorophore, as has previously been suggested (6). Arg⁹⁶ is likely to be essential for the formation of the fluorophore and may help catalyze the initial ring closure. Finally, Tyr¹⁴⁵ shows a typical stabilizing edge-face interaction with the benzyl ring. Trp⁵⁷, the only tryptophan in GFP, is located 13 to 15 Å from the chromophore, and the long axes of the two ring systems are nearly parallel. This conformation indicates that efficient energy transfer to the latter should occur and explains why no separate tryptophan emission is observable (2, 17).

Although the electron density map is for the most part consistent with the proposed structure of the chromophore (2, 7) in the *cis* [2-1 configuration, with no evidence for any substantial fraction of the opposite isomer around the chromophore double bond, difference features are found at $>4\sigma$ in the final ($F_o - F_c$) electron density map that can be interpreted to represent either the intact, uncyclized polypeptide or a carbinolamine (Fig. 2C, inset). This interpretation suggests that a significant fraction, perhaps as much as 30% of the molecules in the crystal, has failed to undergo the final dehydration reaction. Confirmation of incomplete dehydration comes from electrospray mass spectrometry, which consistently shows that the average masses of both wild-type and S65T GFP (31,086 ± 4 and 31,099.5 ± 4 daltons, respectively) are 6 to 7 daltons higher than predicted (31,079 and 31,093 daltons, respectively) for the fully matured proteins. Such a discrepancy could be explained by a 30 to 35% mole fraction of apoprotein or carbinolamine with 18 or 20 daltons higher molecular size (18). Mutants of GFP that increase the efficiency of fluorophore matu-

Table 1. Summary of GFP structure determination. Data were collected at room temperature in-house with either Molecular Structure Corporation R-axis II or San Diego Multiwire Systems (SDMS) detectors (Cu Kα) and later at beamline X4A at the Brookhaven National Laboratory at the selenium absorption edge (λ = 0.979 Å) with image plates. Data were evaluated by use of the HKL package (25) or the SDMS software (26). Each data set was collected from a single crystal. Heavy-atom soaks were 2 mM in mother liquor for 2 days. Initial electron density maps were based on three heavy-atom derivatives with the use of in-house data, then later were replaced with the synchrotron data. The EMTS (ethylmercurithiosalicylate) difference Patterson map was solved by inspection, then used to calculate difference Fourier maps of the other derivatives. Lack of closure refinement of the heavy-atom parameters was performed with the Protein package (27). The multiple isomorphous replacement (MIR) maps were much poorer than the overall figure of merit would suggest, and it was apparent that the EMTS isomorphous differences dominated the phasing. The enhanced anomalous occupancy for the synchrotron data provided a partial solution to the problem. The phasing power was reduced for the synchrotron data, but the figure of merit was unchanged. All experimental electron density maps were improved by solvent flattening with the program DM of the CCP4 (28) package, assuming a solvent content of 38%. Phase combination was performed with PHASCO2 of the Protein package with a weight of 1.0 on the atomic model. Heavy-atom parameters were subsequently improved by refinement against combined phases. Model building proceeded with FRODO and O (29), and crystallographic refinement was performed with the TNT package (30). Bond lengths and angles for the chromophore were estimated with CHEM3D (Cambridge Scientific Computing). Final refinement and model building were performed against the X4A selenium data set, with (2F_o - F_c) electron density maps. The data beyond 1.9 Å resolution have not been used at this stage. The final model contains residues 2 to 229 as the terminal residues are not visible in the electron density map, and the side chains of several disordered surface residues have been omitted. Density is weak for residues 156 to 158, and coordinates for these residues are unreliable. This disordering is consistent with previous analyses showing that residues 1 and 233 to 238 are dispensable but that further truncations prevent fluorescence (12). The atomic model has been deposited in the Protein Data Bank (access code 1EMA).

Crystal	Resolution (Å)	Total observed	Unique observed	Compl. (%) ^a	Compl. (shell) ^b	R _{merge} (%) ^c	R _{iso} (%) ^d
<i>Diffraction data statistics</i>							
R-axis II							
Native	2.0	51,907	13,582	80	69	4.1	5.8
EMTS	2.6	17,727	6,787	87	87	5.7	20.6
SeMet	2.3	44,975	10,292	92	88	10.2	9.3
Multiwire							
HG14-Se	3.0	15,380	4,332	84	79	7.2	28.8
X4A							
SeMet	1.8	126,078	19,503	80	55	9.3	9.4
EMTS	2.3	57,812	9,204	82	66	7.2	26.3
Derivative	Resolution (Å)	Number of sites	Phasing power ^f	Phasing power (shell)	FOM ^g	FOM (shell)	
<i>Phasing statistics</i>							
In-house							
EMTS	3.0	2	2.08	2.08	0.77	0.72	
SeMet	3.0	4	1.66	1.28	—	—	
HG14-Se	3.0	9	1.77	1.90	—	—	
X4A							
EMTS	3.0	2	1.36	1.26	0.77	0.72	
SeMet	3.0	4	1.31	1.08	—	—	
<i>Atomic model statistics</i>							
Protein atoms							1,790
Solvent atoms							94
Resolution range (Å)							20–1.9
Number of reflections (F > 0)							17,676
Completeness							84
R factor ^h							0.175
Mean B value (Å ²)							24.1
<i>Deviations from ideality</i>							
Bond lengths (Å)							0.014
Bond angles (°)							1.9
Restrained B values (Å ²)							4.3
Ramachandran outliers							0

^aCompleteness is the ratio of observed reflections to theoretically possible reflections expressed as a percentage. ^bShell indicates the highest resolution shell, typically 0.1 to 0.4 Å wide. ^cR_{merge} = $\sum |I - \langle I \rangle| / \sum I$, where $\langle I \rangle$ is the mean of individual observations of intensities. ^dR_{iso} = $\sum |F_o - F_c| / \sum F_o$. ^eDerivatives were EMTS (ethylmercurithiosalicylate; residues modified Cys⁶⁶ and Cys⁷⁰), SeMet (selenomethionine-substituted protein; Met¹ and Met²³³ could not be located), and HG14-SeMet (double derivative HG14 on a SeMet background). ^fPhasing power = $(F_o)/\langle E \rangle$, where $\langle E \rangle$ = root mean square heavy-atom scattering and $\langle E \rangle$ = lack of closure. ^gFOM, mean figure of merit. ^hStandard crystallographic R factor; R = $\sum |F_o - F_c| / \sum F_o$.

Table 2. Spectral properties of Thr²⁰³ mutants compared to S65T (10). The mutations F64L, V68L, and S72A improve the folding of GFP at 37°C (37) but do not significantly shift the emission spectra.

Clone	Mutations	Excitation maximum (nm)	Extinction coefficient (10 ³ M ⁻¹ cm ⁻¹)	Emission maximum (nm)
S65T	S65T	489	39.2	511
5B.9B	T203H/S65T	512	19.4	524
6C	T203Y/S65T	513	14.5	525
10B	T203Y/F64L/S65G/S72A	513	30.8	525
10C	T203Y/S65G/V68L/S72A	513	36.5	527
11	T203W/S65G/S72A	502	33.0	512

systems would lower the energy of the excited state of the adjacent chromophore. All three substitutions did indeed shift the emission peak to >520 nm (Table 2). A particularly attractive mutation was T203Y/S65G/V68L/S72A, with excitation and emission peaks at 513 and 527 nm, respectively. These wavelengths are sufficiently different from those of previous GFP mutants to be readily distinguishable by appropriate filter sets on a fluorescence microscope. The extinction coefficient, 36,500 M⁻¹ cm⁻¹, and quantum yield, 0.63, are almost as high as those of S65T (10).

Comparison of *Aequorea* GFP with other protein pigments is instructive. Unfortunately, its closest characterized homolog, the GFP from the sea pansy *Renilla reniformis* (1, 6), has not been sequenced or cloned, although its chromophore is derived from the same FSYG sequence as in wild-type *Aequorea* GFP (22). The closest analog for which a three-dimensional structure is available is the photoreceptor protein (PYP) (23), a 14-kD photoreceptor from halophilic bacteria. PYP in its native dark state absorbs maximally at 446 nm and transduces light with a quantum yield of 0.64, rather closely matching wild-type GFP's long-wavelength absorbance maximum near 475 nm and fluorescence quantum yield of 0.72 to 0.85. The fundamental chromophore in both proteins is an anionic *p*-hydroxynaphthyl group, which is covalently attached to the protein via a thioether linkage in PYP and a heterocyclic iminolactam in GFP. Both proteins stabilize the negative charge on the chromophore with the help of buried cationic arginine and neutral glutamic acid groups.

Arg⁵² and Glu⁴⁶ in PYP and Arg⁹⁶ and Glu¹²² in GFP, although in PYP the residues are close to the oxyphenyl ring whereas in GFP they are nearer the carbonyl end of the chromophore. However, PYP has an overall α/β fold with appropriate flexibility and signal transduction domains to enable it to mediate the cellular phototactic response, whereas GFP has a much more regular and rigid β barrel to minimize parasitic dissipation of the excited state energy as thermal or conformational motions. GFP provides an elegant example of how a visually appealing and extremely useful function—efficient fluores-

cence—can be spontaneously generated from a cohesive and economical protein structure. *Note added in proof:* An independent determination of the structure of wild-type GFP has been carried out by Yang et al. (24).

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- Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; R. Heim, A. B. Cubitt, R. Y. Tsien, *Nature*, **373**, 664 (1995).
- This mutant also contains the ubiquitous Q80R substitution, which accidentally occurred in the early distribution of the GFP cDNA and is not known to have any effect on the protein properties (3). Histidine-tagged S65T GFP (10) was overexpressed in the plasmid JM109/PRSET_g in 4 liters of YT broth plus ampicillin at 37°C, stirred at 450 rpm and 5 liter/min air flow. The temperature was reduced to 25°C at an absorbance at 595 nm (A_{595}) of 0.3, followed by induction with 1 mM isopropyl- β -thiogalactoside for 5 hours. Cell paste was stored at –80°C overnight and then resuspended in 50 mM Hepes (pH 7.9), 0.3 M NaCl, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), passed once through a French press at 68,000 kPa, then canistered at 48,400g for 45 min. The supernatant was applied to a Ni-nitrilotriacetic acid (NTA) agarose column (Clagene), followed by washing with 20 mM imidazole, then eluted with 100 mM imidazole. Green fractions were pooled and subjected to chromatographic (Sigma) proteolysis (1:50 w/w) for 22 hours at room temperature. After addition of 0.5 mM PMSF, the digest was reapplied to the Ni column. NH₄-terminal sequencing verified the presence of the correct NH₂-terminal methionine. After dialysis against 20 mM Hepes (pH 7.5) and concentration to $A_{280} = 20$, rod-shaped crystals were obtained at room temperature in hanging drops containing 5 μ l of protein and 5 μ l of well solution, 22 to 26% polyethylene glycol (PEG 4000, Serva), 50 mM Hepes (pH 8.0 to 8.5), 50 mM MgCl₂ and 10 mM 2-mercaptoethanol within 5 days. Crystals were 0.05 mm across and up to 1.0 mm long. The space group is P2₁2₁2₁, with $a = 51.8$, $b = 62.8$, $c = 70.7$ Å, $Z = 4$. Two crystal forms of wild-type GFP, unrelated to the present form, have been described by M. A. Perrozo, K. B. Ward, R. B. Thompson, and W. W. Ward [*J. Biol. Chem.*, **263**, 7713 (1988)].
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- The natural abundance of ¹³C and ²H and the finite resolution of the Hewlett-Packard 5989B electrospray mass spectrometer used to make these measurements do not permit the individual peaks to be resolved, but instead yield an average mass peak with a full width at half maximum of 55 daltons. The molecular sizes shown include the His-tag, which has the sequence MRGSH-HHH-HGMASMTG-GGGMGRDLYDDDKDPAAEF (9, 10).
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